THE DETERMINATION OF CREATININE AND CREATINE IN BLOOD AND URINE WITH THE PHOTOELECTRIC COLORIMETER

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In spite of their biological importance, measurement of creatinine and creatine in blood plasma has been uncertain because of the low concentrations in which these substances, especially creatine, appear, and the non-specificity of the Jaffe reaction commonly employed for their determination. The presence of creatine in plasma has been questioned since the work of Wilson and Plass (1), because the increment of color after hydrolysis is equivocal when analyzed in visual colorimeters. For this reason Wu (2) concluded that the blood of normal adult males contains no creatine. It seemed possible that by the use of the photoelectric colorimeter, which is peculiarly adapted to the measurement of small changes in the color of faintly tinted solutions, greater accuracy in the measurement of creatinine might be attained and, thereby, that the presence of creatine might be detected and its concentration measured. Such a procedure has been devised and the presence, in appreciable and measurable concentration, of material that is presumably creatine has been established. The procedure is an adaptation of the Folin and Wu (3) colorimetric technique to the Evelyn-Malloy (4) photoelectric colorimeter.

EXPERIMENTAL

A procedure based on these principles has been employed by Horvath (5) and another is described by the manufacturers of the photoelectric colorimeter. For the calculation of the results the manufacturer proposes the formula \( x = \frac{1000L}{K_2} \), where \( K_2 = 6.9 \) and \( L = 2 - \log C \), in which \( C \) represents the galvanometer reading and \( x \), the creatinine in mg. per cent. The curve obtained by this formula did not, however, agree with the results obtained from the analysis of standard solutions of creatinine and creatine. This is shown in Fig. 1.

For purposes of standardization solutions of creatinine zinc chloride and creatine were used. Both were checked by analysis for nitrogen by the macro-Kjeldahl procedure and in addition it was ascertained that after

1 Rubicon Company, Philadelphia.
hydrolysis the color reaction given by creatine was equal to that given by an equivalent amount of creatinine zinc chloride which had been subjected to identical treatment, including the hydrolysis. Hydrolysis was effected by autoclaving the solutions at 15 pounds pressure for 20 minutes.

In the original Folin and Wu technique a saturated solution of picric acid was used. In some earlier studies Welt found that it was impossible to duplicate in summer the curves he had defined in the winter with standard creatinine solutions. This he attributed to the fact that the solubility of picric acid varied greatly with temperature (a saturated solution contains 12.2 gm. per liter at 20°, 63.3 gm. at 100°). This was verified in the present study. Therefore, instead of a saturated solution, a solution containing 11.75 gm. of picric acid per liter was adopted. This is just sufficiently unsaturated to avoid any danger of precipitation at ordinary laboratory temperatures. It is advisable to keep the solution stoppered in a comparatively cool place to prevent evaporation.

With this modification it proved feasible to measure creatinine in serum with ease and accuracy. By means of standard solutions of creatinine zinc chloride, a curve was drawn which is depicted in the top curve of Fig. 1. This curve has been repeatedly checked for 4 years upon one colorimeter by means of standard solutions and has proved constant.

2 Welt, L. G., unpublished studies (1937).
On the other hand, it proved inapplicable to a second instrument, ostensibly identical. It is, therefore, necessary to construct a calibration curve for each instrument. It will be noted that the curve plotted on semilogarithmic paper is rectilinear within the limits of accuracy of the procedure with concentrations of creatinine only up to 5 mg. per cent. Above this it diverges perceptibly from linearity. This divergence has been repeatedly verified on two instruments. The reason for the curvature can only be conjectured. It is suspected that it is referable to the bicolorimetric character of the determination (as the color produced by creatinine increases, the color from the alkaline picrate decreases) and the fact that the spectral interval of the filter does not permit precise enough discrimination between the two colors. This curvature limits readings to the range of 1 to 5 mg. per cent in 1:10 filtrates. This spans, however, the most accurate reading range of the colorimeter.

In the original technique of Folin and Wu, for creatine, since hydrochloric acid is added as a dehydrating agent, readings are finally made in a 1:20 dilution, only one-half as concentrated as that used for creatinine. Since normal serum may contain as little as 1 mg. per cent of creatinine and only a fraction of 1 mg. per cent of creatine, it is obvious from the calibration curve of Fig. 1 that this would bring the readings for creatinine and creatine into the range in which the colorimeter is insensitive. With 1:10 filtrates creatinine can be read accurately only in concentrations of 1 to 5 mg. per cent. An attempt was, therefore, made to use 1:5 tungstic acid filtrates in which the ratio of serum to tungstate was the same as it was in the conventional 1:10 filtrates. The lowest curve in Fig. 1 describes the reading on 1:5 filtrates. It will be seen that the object was not achieved by this method, since readings can be made accurately only in the range of 2 to 5 mg. per cent. This would be equivalent to 1 to 2.5 mg. per cent in 1:10 filtrates. It will be noted that the curve is again rectilinear only over this range. It was established that the concentrations of tungstic acid could be varied 100 per cent without affecting the color developed in the Jaffe reaction. It is, therefore, permissible, if the concentration of creatinine in serum is greater than 5 mg. per cent, to double the dilution with water at any stage of the procedure to bring the colorimetric readings within the practicable range of the instrument.

In an effort to discover whether the tungstic acid had any effect on color development, blanks and standards made up in solutions of tungstate of the concentrations found in plasma filtrates were compared with blanks and standards made up in distilled water. In the creatinine procedure tungstate proved to have no effect on the intensity of the color developed. When the same test was applied to the creatine procedure, a precipitate formed during autoclaving in all tungstate filtrates to which hydrochloric
acid was added. This was insoluble in alkaline picrate, rendering readings meaningless, owing to interference with the transmission of light by the precipitate. No such precipitate formed in filtrates of plasma subjected to the same procedure, presumably because the plasma contained sufficient buffer to modify the effect of the acid. To obviate this difficulty smaller amounts of acid were added. This led to the discovery that at autoclave temperatures, in tungstate filtrates, creatine was dehydrated completely without the addition of acid. This simplified the procedure and at the same time eliminates the necessity of determining creatine and creatinine in different dilutions. The completeness of dehydration and the accuracy with which creatine can be determined are both illustrated in Fig. 1, in which the circles representing measurements of creatine fall sharply on the 1:10 creatinine curve. It is also evident from this coincidence that the color produced by creatinine is not modified by autoclaving.

Method

The analytical procedure finally adopted is essentially that of Folin and Wu (3) adapted to the Evelyn colorimeter. Only two important modifications have been introduced: the use of a more dilute picric acid solution and the omission of hydrochloric acid for the dehydration of creatine. The same method has been adapted to the analysis of urine.

Preparation of Urine—Folin’s (6) original methods for the determination of urinary creatinine and creatine were designed to take advantage of the high concentrations of these compounds in urine and the consequently deeper colors they yielded with alkaline picrate. Such deep colors are, however, unsuited to the photoelectric colorimeter. In attempts to apply the Evelyn instrument to the original technique it was found that standard creatinine solutions yielded inconsistent results. As the photoelectric colorimeter is most accurate with low concentrations of color, and because of the obvious advantages of using the same technique for both blood and urine, the method evolved for analysis of serum has been applied directly to urine.

The variability of concentrations of creatinine and creatine in urine presents obvious difficulties, since the range in which colors can be read is so restricted. This obstacle was surmounted by Tierney3 by the expedient of diluting all urines to the same specific gravity. The following procedure has, in the great majority of instances, yielded concentrations of creatinine and creatine within the limits which can be read with the colorimeter.

The urine, whether a 24 hour or a fractional specimen, is first measured (V), then diluted to a specific gravity of 1.010, and again measured (Vd).

3 Tierney, N. A., to be published.
Of the diluted urine, 5 cc. are again diluted to 100 cc. in a volumetric flask. If the specific gravity of the original urine was less than 1.010, the first dilution is omitted and an aliquot appropriately greater than 5 cc. is diluted to 100 cc. in the volumetric flask. The urine thus finally diluted is analyzed by the procedure described below.

**Reagents—**
1. An N/12 sulfuric acid solution.
2. A 10 per cent solution of sodium tungstate.
3. A picric acid solution, each liter of which contains 11.75 gm. of picric acid which has been purified according to the directions of Folin and Wu (3).
4. A carbonate-free 10 per cent solution of sodium hydroxide.

**Analytical Procedure—** For duplicate measurements of both creatine and creatinine, 5 cc. of serum or of the diluted urine are placed in a flask, followed by 40 cc. of N/12 sulfuric acid and 5 cc. of sodium tungstate solution. After thorough shaking the mixture is filtered (in urines containing no protein in which no precipitate appears filtration may be omitted). Of the protein-free tungstic acid filtrate, 8 cc. are measured into each of four of the special colorimeter tubes, while 8 cc. of water are measured into a fifth tube to serve as a blank. The mouths of two of the four tubes containing filtrate are covered with tin-foil and the tubes are autoclaved at 15 pounds pressure (115–120”) for 20 minutes. At the end of this period the tubes are removed from the autoclave and allowed to cool. The alkaline picrate solution is then prepared by adding 1 volume of 10 per cent sodium hydroxide to 5 parts of the picric acid solution. The alkaline picrate solution should be made up not longer than 5 minutes before it is to be used, as a flaky precipitate forms in it rather rapidly. Although this dissolves readily when the picrate is added to the tungstate filtrate, it makes proper measurement of the solution difficult.

To the contents of each of the five tubes (the two which have been autoclaved, the two containing unautoclaved filtrate, and the water blank) are added 4 cc. of the alkaline picrate solution. The tubes are set aside for 20 minutes to permit complete development of color, before colorimetric readings are made. Readings need not be made immediately after this interval, however, as the color remains unchanged for at least 2½ hours. After the color has been developed, the readings are made in the colorimeter with a 520 μμ filter (transmission 495 to 550 μμ; Rubicon glasses Nos. 3522, 4306, and 5032). The blank tube is first introduced and, with this in place, the galvanometer is set at 100. The colors of the other tubes are then read in the usual manner.

**Calculations—** The galvanometer readings are converted to terms of creatinine by interpolation on a curve constructed by plotting readings
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obtained from standard solutions of creatinine zinc chloride (7). Reagents should be checked from time to time by analyses of standard solutions. If creatine solutions are employed, the determinations must be made on the day the solutions are prepared, because creatine solutions are quite unstable. The curve for creatinine is conveniently graduated in terms of mg. per cent of creatinine in the original specimen. The difference between the readings of the autoclaved and unautoclaved specimens represents creatine in terms of creatinine. This is converted to creatine by multiplying by the factor 1.16.

**Table I**

*Creatinine and Creatine Added to Serum*

<table>
<thead>
<tr>
<th>Added</th>
<th>Recovery</th>
<th>Error</th>
<th>Total error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (1.35 mg. per cent originally)</td>
<td>Creatine* (0.89 mg. per cent originally)</td>
<td>Creatinine</td>
<td>Creatine</td>
</tr>
<tr>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>3.25</td>
<td>4.39</td>
</tr>
<tr>
<td>0</td>
<td>1.9</td>
<td>1.50</td>
<td>3.95</td>
</tr>
</tbody>
</table>

* Expressed as creatinine.
† Expressed as creatine + creatinine.

The following formulas may be used to calculate the concentrations and amounts of creatinine and creatine in urine,

\[
\frac{V_d \times 100 \times C}{V \times a} = X = \text{mg. creatinine in 100 cc. of original specimen}
\]

\[
\frac{V \times X}{100} = \text{gm. creatinine in total specimen of urine}
\]

where \(a\) = the aliquot of the diluted urine, in cc.; \(V\) = the volume of the undiluted urine in liters; and \(V_d\) = the volume to which the urine was diluted to bring its specific gravity to 1.010.

If the reading of creatinine (or creatine + creatinine, expressed in terms of creatinine) on the curve exceeds 5 mg. per cent, in the analysis of plasma or urine either a smaller aliquot must be taken or the tungstic acid filtrate must be diluted with water before analysis. Ordinary 1:10 or even 1:5 filtrates, diluted with equal volumes of water, give readings that are proportional to the dilution.

The total error of colorimeter readings, as judged by the analysis of standard solutions of creatinine and of creatine + creatinine, is slightly less than 4 per cent over the range of 1 to 5 mg. per cent, as is evident from
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the data represented in Fig. 1. The error in measurement of creatine added to creatinine is somewhat greater, amounting at times to as much as 10 per cent, because, since creatine is measured by difference, it incurs a double error. Creatinine added to human sera may be recovered quantitatively. The instability of creatine renders its recovery somewhat less reliable. However, in analyses after such additions, at least 80 per cent of creatine was recovered as creatine, and at least 95 per cent as creatinine + creatine. An example of such an analysis is given in Table I. The error, within concentrations thus far encountered in serum, does not exceed 0.1 mg. per cent.

A series of analyses of individual and pooled human sera gave values of 0.9 to 1.7 mg. per cent for creatinine. A small series of analyses of normal human sera gave values averaging 0.3 mg. per cent for creatine. In pooled sera from the clinical laboratory as much as 1.1 mg. per cent was found. Analyses of urine of normal adult males yielded values for creatinine within the generally accepted limit. No creatine could be detected in such urines, although it was found in certain pathological urines. This is at variance with the reports of Dill and Horvath (8).

The method has been employed for a year in the chemical laboratory of the Department of Internal Medicine of the Yale University School of Medicine and has given consistent results.

**DISCUSSION**

Though the presence of creatinine in the blood serum of humans had been definitely established or some time (9–12), creatine was present, if at all, in such low concentrations that its determination was impossible by optical colorimetry. Therefore, the availability of a method sufficiently sensitive to detect the presence of definite quantities of "apparent creatine" should open up avenues of exploration in a hitherto inaccessible field. Since a large percentage of the chromogens present in the cells of blood is presumably not creatinine (11), no attempts have been made as yet to apply the method to whole blood. At the present time definite evidence that the serum chromogens obtained by hydrolysis of tungstate filtrates are actually creatine is lacking, and the application of enzymatic methods to blood such as have been employed in tissues (13) is to be desired. However, presumptive confirmatory evidence that the "apparent creatine" found in the serum by this method actually is creatine has been recently furnished in this laboratory by the discovery that its concentration in persons with creatinuria is definitely elevated.

The urine method presented here does not represent a theoretical advance from previous procedures employing optical colorimeters (6), but does possess the advantage of simplicity and somewhat increased accuracy.
SUMMARY

The Jaffe reaction for the determination of serum creatinine is adapted for use with the Evelyn-Malloy photoelectric colorimeter, with a constant strength of picric acid solution.

In the measurement of creatine, conversion to creatinine is accomplished by autoclaving without the use of hydrochloric acid acidification.

The method can be applied to the analysis of urines, with the same reagents and calibration curve that are used in the analysis of serum.

The total colorimetric error is 4 per cent of theoretical.

Creatinine added to serum is recovered quantitatively.

0.9 to 1.7 mg. per cent of creatinine was found in normal and pooled human sera.

Definite amounts of creatine, averaging 0.3 mg. per cent, were found in normal human sera.

The amounts of creatinine and creatine found in urine were consistent with the findings of other observers.

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